SUPPLEMENTAL MATHEMATICAL APPENDICES

Supplemental Appendix 1: Blind-Source Separation

Signal artifacts due to brain motion and hemodynamics are commonplace in physiologic measurements of aggregate neural activity. Numerous computational approaches exist for removing broadband and single frequency noise from electroencephalography (EEG), functional magnetic resonance imaging (fMRI) and intrinsic optical measurements, including approaches based on principal component analysis, independent component analysis (ICA), frequency-domain filtering, direct estimation and subtraction of periodic artifacts in the time domain, and wavelet decomposition (Addison, 2005; Akemann et al., 2012; Feldman, 2011; Grouiller et al., 2007; Huang and Shen, 2014; Jung et al., 2001; Mitra and Pesaran, 1999; Niazy et al., 2005). However, these approaches have generally been optimized for specific experimental paradigms and noise sources, and do not generalize well to other measurement modalities and paradigms.

To remove physiologic artifacts from our optical voltage recordings, we sought a principled approach to blind source separation. We began by constructing a quantitative model of the expected sources of signal and artifacts in the recordings, including both broadband and frequency-specific noise sources. We then designed a two-stage unmixing procedure that incorporates the structure of the noise in each of the two detection channels and yields an unmixing that is near shot-noise limited in its performance (**Figure 2**). The algorithm's first stage provides an empirical estimate of the time-dependence of cardiovascular pulsations, based on data from the optical reference channel. The second stage uses ICA to unmix the two fluorescence time traces plus the estimated cardiovascular time trace into three decontaminated records of the trans-membrane voltage signals, cardiovascular artifacts and broadband artifacts.

We describe the fluctuations observed in the red, $r(t)$, and green, $g(t)$, fluorescence traces (**Figure 1C**) as arising from neural signals, $S(t)$, motion artifacts, $A(t)$, hemodynamic artifacts, $H(t)$, fluctuations in the laser's mean power, $L(t)$, and fluctuations due to photon shot

noise and photodetector electronic noise in the two emission channels (approximated as stationary Gaussian distributions with standard deviations, σ_r and σ_g):

$$g(t) = g_0[1 + S(t) + A(t) + H(t)] \cdot [1 + \alpha L(t)] + \mathcal{N}(0, \sigma_g)$$

$$r(t) = r_0[1 + \delta S(t) + \lambda_1 A(t) + \lambda_2 H(t)] \cdot [1 + \alpha L(t)] + \mathcal{N}(0, \sigma_r) \quad .$$

Here g_0 and r_0 are the mean fluorescence intensities of the two color channels, δ models the extent of the crosstalk between the two channels, λ_1 and λ_2 are the ratios of the magnitudes of the physiological artifacts in the two channels, and α accounts for differences in the magnitudes of the non-stationary fluctuations as observed in the direct measurements of laser power and those in the resulting fluorescence traces. These parameters varied considerably between mice due to differences in the relative intensities of the two fluorescence channels. δ ranged between 0.001–0.1, depending on the relative emission power in the two channels. λ_1 and λ_2 ranged between 0.2–2 due to differences in the fluorescence baseline values of the two channels and due to spectral differences in light absorption by hemoglobin. α was generally between 0.2–1 due to differences in photodiode filter properties, lock-in amplifier settings and the intensity of laser fluctuations between the photodiode and fiber patch cord.

The variances of these fluctuations were small compared to unity: $\mathrm{Var}[S(t)], \mathrm{Var}[L(t)], \mathrm{Var}[A(t)], \mathrm{Var}[H(t)] \sim 0.01$. One can therefore ignore products of the perturbations to approximate:

$$g(t) = g_0[1 + S(t) + A(t) + H(t) + \alpha L(t)] + \mathcal{N}(0, \sigma_g)$$

$$r(t) = r_0[1 + \delta S(t) + \lambda_1 A(t) + \lambda_2 H(t) + \alpha L(t)] + \mathcal{N}(0, \sigma_r) \quad .$$

ICA applied to $g(t)$ and $r(t)$ determines a weight vector W that maximizes the non-Gaussianity of the projected fluorescence traces. This will yield statistically independent sources $c_1(t) = W_{11} g(t) + W_{12} r(t)$ and $c_2(t) = W_{22} g(t) + W_{22} r(t)$ (Hyvarinen and Oja, 2000). The fluctuations $S(t), A(t), H(t)$ and $L(t)$ are non-Gaussian, and likely statistically independent.

Thus, in the noise-free case with $\lambda_1 = \lambda_2 = 1$, ICA should recover sources of the form $c_1(t) \propto S(t); c_2(t) \propto A(t) + H(t) + \alpha L(t)$, because putative sources that mix $S(t)$ and physiological artifacts would not be statistically independent (Lukacs, 1954).

We first attempted to unmix traces using ICA alone, as recordings in mice expressing the control fluorophores YFP and mCherry showed strong correlations between the green and red fluorescence channels, suggesting there were approximately equal contributions in each color channel from brain motion and hemodynamic noise sources (**Figures S1, S2**). Although ICA alone was effective in some mice for removing both motion and blood flow artifacts, in other mice some hemodynamic artifacts remained in the unmixed signal channel, seemingly due to an unequal apportionment of the two artifacts between the two color channels.

Most versions of ICA are ill suited for under-determined problems in which the number of sources exceeds the number of signals, limiting their utility for extraction of more than two sources from two traces. Numerous extensions of ICA exist that do allow unmixing of more sources than signals, typically by projecting the recorded traces onto a large basis set, such as a Fourier transform or wavelet basis, in which the sources are hypothesized to be sparse (Bofill, 2001; Lewicki and Sejnowski, 2000; Yilmaz, 2004). Unfortunately, our traces contained broadband motion artifacts, narrowband hemodynamic artifacts, and substantial photon shot noise, which precluded a straightforward application of one of the established projection methods based on a sparseness assumption.

Hence, we estimated the narrowband hemodynamic fluctuations directly from the red fluorescence reference trace, and we used this as a separate input into the ICA. We modeled hemodynamic noise as a sinusoid with time-varying amplitude, phase, and frequency: $h(t) = a(t)\sin[2\pi f(t) \cdot t + \phi(t)]$. If desired, this model can be extended to incorporate the harmonics of hemodynamic noise at integer multiples of the heartbeat frequency (**Figure 2B**).

We estimated amplitude, frequency and phase parameters from the red fluorescence trace by dividing the trace into time bins of 2 s each, and performing a least-squares curve fit between $r(t)$ and $h(t)$ in each bin, with no constraints between the parameter values in successive bins. We then simultaneously unmixed $g(t)$, $r(t)$, and $h(t)$ using ICA and denoted the signal trace as $c_1(t) = W_{11}g(t) + W_{12}r(t) + W_{13}h(t)$, which we designated as the trace with the largest relative proportion of green fluorescence. To preserve the shot-noise statistics and the magnitude of fluorescence changes in the unmixed traces, we normalized the rows of the mixing matrix W such that $W_{11} = W_{22} = W_{33} = 1$.

In cases when expression of the red reference fluor was dim, we used both the 488-nm- and the 561-nm-wavelength laser light sources. We modulated the amplitudes of the two lasers 90 degrees out of phase with each other and continuously monitored their emission powers, $P_g(t)$ and $P_r(t)$, respectively. Ignoring the products of perturbations as above, and dividing each optical signal by its mean intensity, we find:

$$g'(t) = 1 + S(t) + A(t) + \alpha_g L_g(t) + \mathcal{N}(0, \sigma_g')$$

$$r'(t) = 1 + A(t) + \alpha_r L_r(t) + \mathcal{N}(0, \sigma_r')$$

$$P_g'(t) = 1 + L_g(t)$$

$$P_r'(t) = 1 + L_r(t)\,.$$

Here the prime index denotes signals normalized by their mean intensity, *e.g.* $g'(t) = g(t)/g_0$. We allowed for a linear scaling of laser power fluctuations by the constants, α_g and α_r, to reflect possible differences in photodiode filter properties, lock-in amplifier settings and the magnitude of laser power fluctuations. We applied ICA similarly to as above, which yielded the resulting signal vector $c_1'(t) = W_{11}g'(t) + W_{12}r'(t) + W_{13}P_g'(t) + W_{14}P_r'(t)$. In these

cases, performing a separate estimation of a hemodynamic trace did not substantially improve the unmixing performance, likely because of the relatively low power in the reference trace.

To ascertain whether the two-laser approach was superior in cases when the reference fluor was expressed brightly, we also compared the performances of the two-channel and four-channel unmixing approaches using mice expressing red tdTomato and a green FRET-opsin voltage sensor. Using phase-sensitive detection, we simultaneously recorded the phase-orthogonal components of both red and green fluorescence excited by the 488 and 561 nm laser sources, and the power fluctuations of each laser. We tuned the emission powers of the 488 and 561 nm lasers so as to excite comparable levels of fluorescence in the reference channel. This configuration allowed us to perform both two-channel and four-channel unmixing on the measured traces. We computed the Pearson correlation coefficient of the signal traces provided by the two methods (r = 0.88 ± 0.015; N = 8 mice; mean ± s.e.m), and the reductions of the hemodynamic artifact in each signal trace (0.7 ± 0.2% hemodynamic artifact remaining using two-channel unmixing and 0.1 ± 0.5% artifact remaining using four-channel unmixing; mean ± s.e.m), which were not significantly different between the two methods (P = 0.5; Wilcoxon signed-rank test).

These analyses showed that the two methods comparably reduced the known physiological artifacts and produced traces with similar overall characteristics. The slight decrease in performance due to the use of the 561 nm laser source was likely due to the instrumentation used: the fluorescence excited by the 561 nm laser had higher variance (s.d. of red fluorescence: 0.6%, s.d. of the green fluorescence: 0.09%), which almost surely resulted from the higher level of emission noise in the 561 nm laser beam. This laser emission noise is specific to our experimental system and is not a general limitation for the approach (s.d. of the 488 nm laser power: 0.12%; s.d. of 561 nm laser power: 0.41%). Therefore, in our apparatus it was preferable when using the bright reference fluor tdTomato to employ only a single 488 nm

laser source to excite both the signal and the reference channels, so as to reduce the impact of laser noise. However in general, the use of one laser is only feasible if: (i) it generates an adequate reference signal, which in turn requires bright expression of the reference fluor; and (ii) the emission spectra of the two fluors are non-overlapping and do not necessitate a time-domain unmixing strategy.

Therefore, in our apparatus it was desirable to use only a single 488 nm laser source to excite both the signal and reference channels to reduce noise induced by the laser. However, the use of one laser approach is only practical if it generates an adequate reference signal, which in turn requires dense expression of the reference fluor.

Overall, our blind source separation algorithm reduced the noise in our optical recordings by ~10-fold, to levels approaching the fundamental limits due to photon shot-noise as set by quantum mechanics (**Figure 2**). This finding indicates that further algorithmic improvements will not dramatically reduce the levels of artifacts present in our measurements.

Supplemental Appendix 2: Noise modeling

Mathematical variables used in the noise model

Variable	Description
f	Temporal frequency of electrical current measurements
λ	Wavelength of light
$S(f)$	Shot noise spectral density
I	Mean electron current
q	Charge of the electron
$\bar{V}$	Time-averaged photo-voltage signal
g_λ	Photodetector responsivity to a photon of wavelength λ
η_λ	Quantum efficiency of the photodetector for a photon of wavelength λ
E_λ	Energy of a photon of wavelength λ

We modeled the noise power in our recordings as a quadrature sum of photon shot noise, fluctuations in laser emission power, and electronic noise in the photodetectors. We directly measured the fluctuations in laser emission power. The electronic noise equivalent power of the photoreceivers was specified by the manufacturer to be 10 fW/$\sqrt{\text{Hz}}$. This was consistent with the data from control recordings in which no light was incident on the photoreceiver.

To calculate the expected levels of shot-noise in our recordings, we first noted that the one-sided shot noise spectral density, $S(f)$, as a function of temporal frequency, f, that is present in a recording of a stationary-mean electrical current is given by $S(f) = 2qI$, where I is the mean current and q is the electron charge (Horowitz and Hill, 1989). We applied this formula to our trans-impedance amplified measurements of photocurrent: $S(f) = 2\frac{gE}{\eta}\bar{V}$. Here $\bar{V}$ is the mean photo-voltage signal after amplification, g is the responsivity of the photodetector, η is the detector's quantum efficiency, and E is the mean photon energy of the optical signal. The three

parameters: g, η, and E are a function of the wavelength of the incident light. The responsivity $g \propto q\eta/\mathrm{E}$, as well as the quantum efficiency η are specified for our photodetectors by the manufacturer for light of 850 nm. We can calculate the responsivity for another wavelength, λ, using $g_\lambda = g_{850}\,\frac{\eta_\lambda}{\eta_{850}}\,\frac{E_{850}}{E_\lambda}$, where the 850 subscript denotes a value specified at 850 nm. We can therefore calculate the power spectral density of the expected shot noise using:

$$S(f) = 2\,\frac{g_{850}E_{850}}{\eta_{850}}\,\bar{V} \ .$$

To compare this calculated noise power to the empirical recordings of fluorescence signals, we generated time traces consisting of Gaussian noise with a power spectrum matching that calculated for the shot noise, applied a single-pole 75 Hz low-pass Butterworth filter to model the frequency response of the photoreceiver, and then digitally filtered the noise traces in the same manner as for the real optical traces.

After this signal processing, we used the above noise model to estimate a theoretical description of the total levels of noise in our recordings, as contributed by photon shot noise, noise from our photoreceivers, and the noise due to laser power fluctuations (**Figure S2C**). We found that this noise model gave an excellent prediction of the empirically determined total noise (**Figure S2D**), differing on average by only ~20% across a 1-50 Hz bandwidth. Finally, we found that this noise model could be used to generate accurate theoretical predictions of the noise floor of our unmixed data, by taking a linear combination of the noise floors estimated separately for each of the detections channels (**Figure S2E**).

References

Addison, P.S. (2005). Wavelet transforms and the ECG: a review. Physiological measurement *26*, R155-199.

Akemann, W., Mutoh, H., Perron, A., Park, Y.K., Iwamoto, Y., and Knopfel, T. (2012). Imaging neural circuit dynamics with a voltage-sensitive fluorescent protein. J. Neurophysiol. *108*, 2323-2337.

Bofill, P.Z., M. (2001). Underdetermined blind source separation using sparse representations. Signal Processing *81*, 2353-2362.

Feldman, M. (2011). Hilbert transform applications in mechanical vibration (Chichester: Wiley).

Grouiller, F., Vercueil, L., Krainik, A., Segebarth, C., Kahane, P., and David, O. (2007). A comparative study of different artefact removal algorithms for EEG signals acquired during functional MRI. NeuroImage *38*, 124-137.

Horowitz, P., and Hill, W. (1989). The art of electronics, 2nd edn (Cambridge England: Cambridge University Press).

Huang, N.E., and Shen, S.S. (2014). Hilbert-Huang transform and its applications, 2nd edn (Hackensack New Jersey: World Scientific).

Hyvarinen, A., and Oja, E. (2000). Independent component analysis: algorithms and applications. Neural networks : the official journal of the International Neural Network Society *13*, 411-430.

Jung, T.P., Makeig, S., McKeown, M.J., Bell, A.J., Lee, T.W., and Sejnowski, T.J. (2001). Imaging Brain Dynamics Using Independent Component Analysis. Proceedings of the IEEE. Institute of Electrical and Electronics Engineers *89*, 1107-1122.

Lewicki, M.S., and Sejnowski, T.J. (2000). Learning overcomplete representations. Neural computation *12*, 337-365.

Lukacs, E.K., E.P. (1954). A property of the normal distribution. Ann. Math. Statist. *25*, 389-294.

Mitra, P.P., and Pesaran, B. (1999). Analysis of dynamic brain imaging data. Biophysical journal *76*, 691-708.

Niazy, R.K., Beckmann, C.F., Iannetti, G.D., Brady, J.M., and Smith, S.M. (2005). Removal of FMRI environment artifacts from EEG data using optimal basis sets. NeuroImage *28*, 720-737.

Yilmaz, O.R., S. (2004). Blind separation of speech mixtures via time-frequency masking. IEEE T Signal Proces *52*, 1830-1847.